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Conservation and function of the transcriptional regulatory protein Runt

(*Drosophila*/segmentation/*run*/*AML1*/Runt domain)

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ABSTRACT **A phylogenetic approach was used to identify conserved regions of the transcriptional regulator Runt. Alignment of the deduced protein sequences from *Drosophila melanogaster*, *Drosophila pseudoobscura*, and *Drosophila virilis* revealed eight blocks of high sequence homology separated by regions with little or no homology. The largest conserved block contains the Runt domain, a DNA and protein binding domain conserved in a small family of mammalian transcription factors. The functional properties of the Runt domain from the *D. melanogaster* gene and the human *AML1* (acute myeloid leukemia 1) gene were compared *in vitro* and *in vivo*. Electrophoretic mobility-shift assays with Runt/AML1 chimeras demonstrated that the different DNA binding properties of Runt and AML1 are due to differences within their respective Runt domains. Ectopic expression experiments indicated that proteins containing the AML1 Runt domain function in *Drosophila* embryos and that sequences outside of this domain are important *in vivo*.**

The *Drosophila* gene runt (*run*) is a member of a newly identified family of transcriptional regulators (1). *run* was initially characterized because of its role as a pair-rule gene during segmentation (2, 3) and was subsequently found to have roles in two other developmental processes in the fly—sex determination and neurogenesis (4, 5). In each pathway, *run* regulates the expression of other genes. Consistent with a role in transcriptional regulation, the Runt protein is localized to the nucleus (6).

The recent isolation of genes that encode a heterodimeric mammalian transcription factor referred to as PEBP2/CBF has added insight on the function of Runt (1). The name PEBP2/CBF (polyoma enhancer binding protein 2/core binding factor) reflects the ability of this factor to bind to sites in the core enhancers of the polyoma tumor virus and murine type C retroviruses (7–9). In addition, PEBP2/CBF binds to sites in enhancers of several genes expressed in T lymphocytes and is involved in the regulation of T-cell-specific gene expression (10). Purification of PEBP2/CBF revealed it is a heterodimer of two unrelated subunits (α and β) (7). Three different, highly related genes encoding the DNA binding, PEBP2/CBFα subunit have been identified in mammals (10–12). All three contain a region that is highly homologous to a 128-amino acid sequence in the Runt protein. This region, referred to as the Runt domain, is responsible for the DNA binding function of PEBP2/CBFα proteins (10). The Runt domain also mediates the heterodimeric interaction with PEBP2/CBFβ (10). The PEBP2/CBFβ subunit does not bind to DNA but increases the stability of the interaction between the Runt domain and DNA (13).

Although Runt is able to bind to DNA *in vitro*, target binding sites in genes regulated by *run* have not yet been identified. One of the best studied genes regulated by *run* is the pair-rule segmentation gene fushi tarazu (*ftz*). In *run* mutant embryos, *ftz* expression is reduced (14). In contrast, when *run* is ectopically expressed, *ftz* expression is increased (15, 16). Analysis of *ftz* reporter genes indicates that *run* acts on *ftz* through a small sequence element, fDE1, that contains a binding site for the FTZ-F1 family of orphan nuclear receptors (16). The fDE1 element does not have a match to the consensus binding site of the mammalian Runt domain proteins, and no *in vitro* interaction has yet been detected between this element and the Runt protein (C. Tsai and J.P.G., unpublished data). A similar story emerged from analysis of a subset of the effects of *run* overexpression on even skipped (*eve*) and hairy (*h*) genes (15). In summary, these experiments provide no evidence that DNA binding is essential for Runt function and suggest that other mechanisms for transcription regulation need to be considered.

Here we use several approaches to investigate the function of Runt. We cloned and sequenced homologues of *run* from two other *Drosophila* species, *Drosophila pseudoobscura* and *Drosophila virilis*, to identify conserved regions that may be important for function. A region containing the Runt domain was the largest and most conserved block of homology among the three species. Investigation of *in vitro* DNA binding properties of *Drosophila* and mammalian Runt domain proteins shows that the differences in DNA binding can be attributed to amino acid differences in the Runt domain. Finally, comparison of the activity of Runt, AML1 (a human Runt domain protein), and Runt/AML1 hybrid proteins in the *Drosophila* embryo indicates that regions outside the Runt domain are important for *in vivo* function.

MATERIALS AND METHODS

Isolation and Sequencing of *run* Homologues. Library screening, subcloning, and other molecular biology techniques were performed by standard techniques (17). *D. pseudoobscura* and *D. virilis* genomic libraries in λEMBL3 were screened with single-stranded digoxygenin-UTP-labeled RNA probes. The templates for RNA synthesis were subclones of *run* cDNAs (6). Hybridization was done at 50°C in 50% formamide/5× SSC/0.1% *N*-lauroyl sarcosine/0.02% SDS/5% blocking reagent (Boehringer Mannheim). The washes were performed according to the manufacturer's instructions (Boehringer Mannheim) except for the final wash, which was done at 50°C in 1× SSC/0.1% SDS.

Isolated phage DNAs were restriction enzyme mapped and overlapping restriction fragments containing homology to *run* were subcloned into Bluescript (Stratagene) for sequencing. Double-stranded DNA was sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* (18) with ^{35}S-labeled dATP and the United States Biochemical Sequenase kit. For *D. pseudoobscura*, a total of 3875 bp were

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U22357 and U22358).

sequenced. For *D. virilis*, a total of 2749 bases were sequenced. The sequence of the restriction fragments was analyzed by the PCGENE program. In the course of these studies, two minor errors were discovered in the *D. melanogaster* sequence. The first error does not alter the protein sequence but creates a *Not* I restriction site in the coding region. The second correction changes amino acid 152 from tryptophan to valine. This amino acid, which is in the Runt domain, is a valine in both other *Drosophila* species and in the mammalian Runt domain proteins. These corrections have been submitted to the GenBank data base.

Electrophoretic Mobility-Shift Assays. DNA binding reactions were performed in 20 mM Tris·HCl, pH 8/10 mM NaCl/3 mM EGTA/5 mM dithiothreitol/0.05% Nonidet P-40 with 0.1 μg of poly[d(IC)] using 10,000–20,000 cpm of labeled DNA in a total vol of 25 μl. The reaction mixtures were incubated for 10 min at room temperature and for 20 min on ice. Loading buffer [5% (vol/vol) glycerol/2 mM Tris·HCl, pH 8/0.025% xylene cyanol/bromophenol blue] was added, and the DNA–protein complexes were electrophoresed on a 10% polyacrylamide (60:1, acrylamide/bisacrylamide) gel in 0.25× TBE at 200 V for 4 hr at 4°C. The probe used was a 37-bp restriction fragment containing the *A*-element of the polyoma virus enhancer (7, 19).

Protein Expression and Purification. Proteins expressed in bacteria were all made as hexahistidine-tagged fusions by cloning relevant cDNA segments into the pQE30 (Qiagen) expression vector. The Runt–AML1 hybrid proteins were made by inserting PCR amplified portions of coding regions from *AML1* cDNAs (20) into a derivative *run* subclone. For the RAB protein, amino acids 82–224 of *D. melanogaster* Runt were replaced with the corresponding amino acids in AML1. In the RAM hybrid, amino acids 112–224 were replaced with the corresponding amino acids in AML1. Fusion proteins were prepared under denaturing conditions according to the manufacturer's instructions (Qiagen).

***Drosophila* Transformation and Embryo Manipulation.** *hs/AML1* and *hs/RAM* were constructed in the CaSpeR *P*-element transformation vector (21) by replacing the *run* coding sequences of CaSpeR:hsrunt with *AML1* and *RAM* coding fragments. Germ-line transformants were produced by standard procedures using the $y\ w^{67c23}$ strain as a host and pπ25.7wc as the *P*-transposase helper plasmid (22). Seven different *hs/AML1* lines and three *hs/RAM* lines were obtained. Two and three lines, respectively, were examined for their effects. Embryos were collected and heat shock treatments were done as described (15). *In situ* hybridization was performed as described (23).

RESULTS

Cloning and Sequencing of *run* Homologues from Other Species. To identify conserved regions of the Runt protein, we sequenced the homologous genes from *D. pseudoobscura* and *D. virilis*, two species that diverged from *D. melanogaster* some 40–60 million years ago (24). The entire protein coding region, the intron, and the 5′ untranslated leader were sequenced. The location of the transcription and translation start sites of each gene was deduced by comparison with the *D. melanogaster* sequence. The nucleic acid sequence of the protein coding region was 82% identical for *D. pseudoobscura* and 77% identical for *D. virilis* when compared to *D. melanogaster*. There is no significant overall homology in noncoding regions. However, in the 5′ untranslated region there is a 96-bp region centered on the transcription start site that is 81% identical between the three species. Each gene has one intron. There is also a 40-bp region of homology at the beginning of the intron. Conservation of sequences in noncoding regions has also been observed in other segmentation genes (25–27). These conserved noncoding regions are likely to have functional significance, presumably as elements involved in regulating expression.

The deduced sequences of the *D. pseudoobscura* and *D. virilis* proteins are 82% and 78% identical, respectively, to the protein from *D. melanogaster*. A three-way alignment identifies eight blocks of sequence homology that, taken together, account for 398 of the 509 amino acids in the *D. melanogaster* protein (Fig. 1). Within these conserved blocks, there is 92% amino acid identity in all three species. This contrasts with only ≈14% amino acid identity in the regions between the blocks. The length of the nonconserved regions is also more variable between species.

The largest conserved block, which is 95% identical in all three *Drosophila* species, contains the Runt domain. The other seven blocks are smaller and in several cases have features similar to motifs found in other transcriptional regulators. For example, region VI is rich in acidic amino acids similar to transcriptional activation domains (28, 29). Conversely, regions I and IV are alanine-rich, similar to putative repression domains (30–32). The last 5 amino acids of this block are VWRPY, a sequence also present at the C terminus of the mammalian Runt domain proteins (10–12).

DNA Binding Properties of Runt Domain Proteins. The largest region of homology identified above is a 192-amino acid block that contains the entire Runt domain and extends N-terminal and C-terminal to this domain by 14 and 54 amino acids, respectively. Previously, a segment of Runt containing this domain was found to bind to a PEBP2/CBF binding site in the polyoma enhancer (1). DNA binding by the *Drosophila*

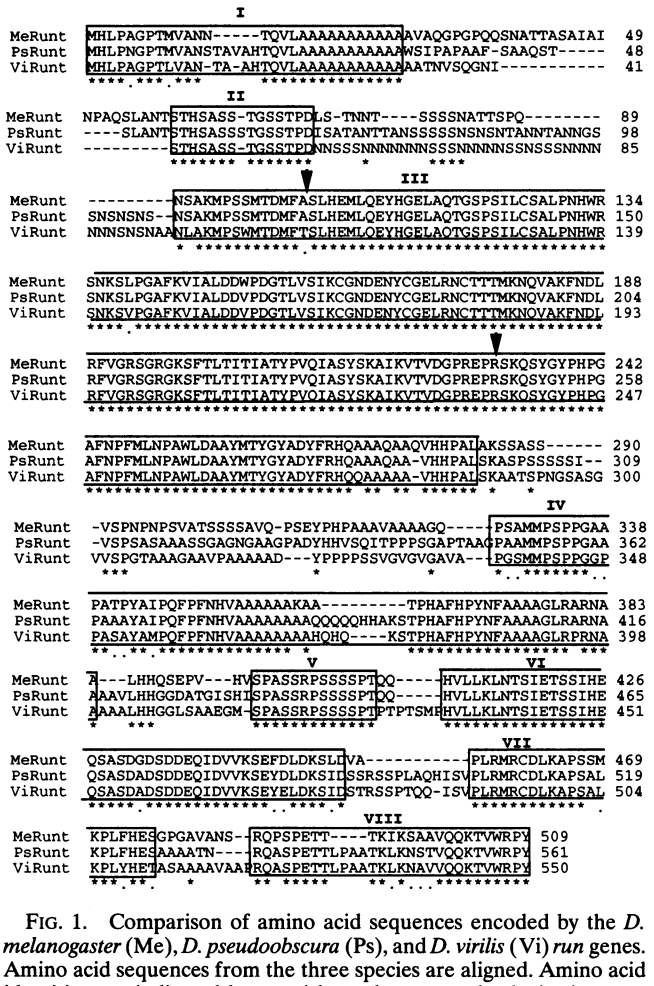

FIG. 1. Comparison of amino acid sequences encoded by the *D. melanogaster* (Me), *D. pseudoobscura* (Ps), and *D. virilis* (Vi) *run* genes. Amino acid sequences from the three species are aligned. Amino acid identities are indicated by asterisks and conserved substitutions are indicated by dots. Gaps are denoted by dashes. Conserved blocks of amino acids are boxed. Arrowheads indicate limits of the Runt domain.

Runt domain was markedly weaker than its mammalian counterparts, especially in the absence of the PEBP2/CBFβ partner protein. To investigate the reasons for this difference, we used electrophoretic mobility-shift assays to compare the DNA binding activities of several Runt protein derivatives. One potential explanation for the difference that is suggested by the conserved flanking regions in the *D. pseudoobscura* and *D. virilis* proteins is that sequences outside of the Runt domain contribute to the DNA binding activity of Runt. However, full-length, bacterially expressed Runt protein behaves similarly to the Runt domain-containing segment used previously; significant DNA binding is detected only in the presence of PEBP2/CBFβ (Fig. 2*B*).

We used two different Runt domain hybrid proteins to further confirm that the weak DNA binding activity of Runt is due to sequences within its Runt domain. The first of these, the RAB hybrid, contains the entire Runt domain from the human AML1 protein in the context of full-length Runt (Fig. 2*A*). Like AML1, but unlike Runt, the RAB protein binds DNA well in both the absence and presence of PEBP2/CBFβ (Fig. 2*B*). The RAB protein contains amino acids N-terminal of its Runt domain that are from AML1. To determine whether these amino acids are responsible for the difference between RAB and Runt, a second hybrid protein, RAM, was tested. The Runt domain of this hybrid is also from AML1 except that the first 7 amino acids are from Runt (Fig. 2*A*). RAM also binds DNA well in both the absence and presence of PEBP2/CBFβ (Fig. 2*B*). Based on these results, we conclude that the different DNA binding properties of Runt and AML1 are due to sequence differences within the Runt domain.

Regulatory Properties of the AML1 Runt Domain in *Drosophila* Embryos. An ectopic expression assay was used to examine the relevance of the different DNA binding properties of Runt and AML1. Previous work showed that transcriptional regulation of the pair-rule segmentation genes *h*, *eve*, and *ftz* is altered in embryos that express a *hs/run* transgene (15). Ectopic expression of the RAM hybrid protein during segmentation alters the patterns of expression of these genes in the same manner as *run*. The effects on *h* and *eve* are stripe specific. For *h*, the repression of stripe 1 is most obvious (Fig. 3 *B* and *C*). Stripes 2 and 5 are also reduced in intensity while stripes 3 and 4 are more intense. For *eve*, the most noticeable difference is the loss of stripe 2 (Fig. 3 *F* and *G*). In addition, stripes 4 and 7 are reduced in intensity and stripes 5 and 6 are not well resolved. The *ftz* gene responds to *hs/run* and *hs/RAM* in a more uniform manner; interstripe repression is lost and the 7 stripes are fused together into one broad band of expression (Fig. 3 *J* and *K*). In some *hs/RAM* lines, the penetrance of these effects is not as strong (Table 1), presumably because of differences in the level of expression. However, these results demonstrate that a Runt protein containing the Runt domain from *AML1* has qualitatively the same regulatory properties as the normal Runt protein in this ectopic expression assay.

In contrast to the above results, AML1 has no effect on the expression patterns of *h*, *eve*, and *ftz* (Fig. 3 *D*, *H*, and *L*; Table 1). Western blot experiments indicate that the amounts of protein produced by the *hs/AML1* transgene are comparable to the levels produced by *hs/run* and *hs/RAM* (data not shown). Thus, differences in the efficiency of protein synthesis or stability do not account for the complete inactivity of AML1 in this assay. This indicates that ectopic expression of the Runt domain alone is not sufficient to alter transcriptional regulation of these *Drosophila* segmentation genes. Taken together, these results indicate that regions outside the Runt domain are critical for the regulatory effects that Runt exerts on the expression of these other pair-rule genes.

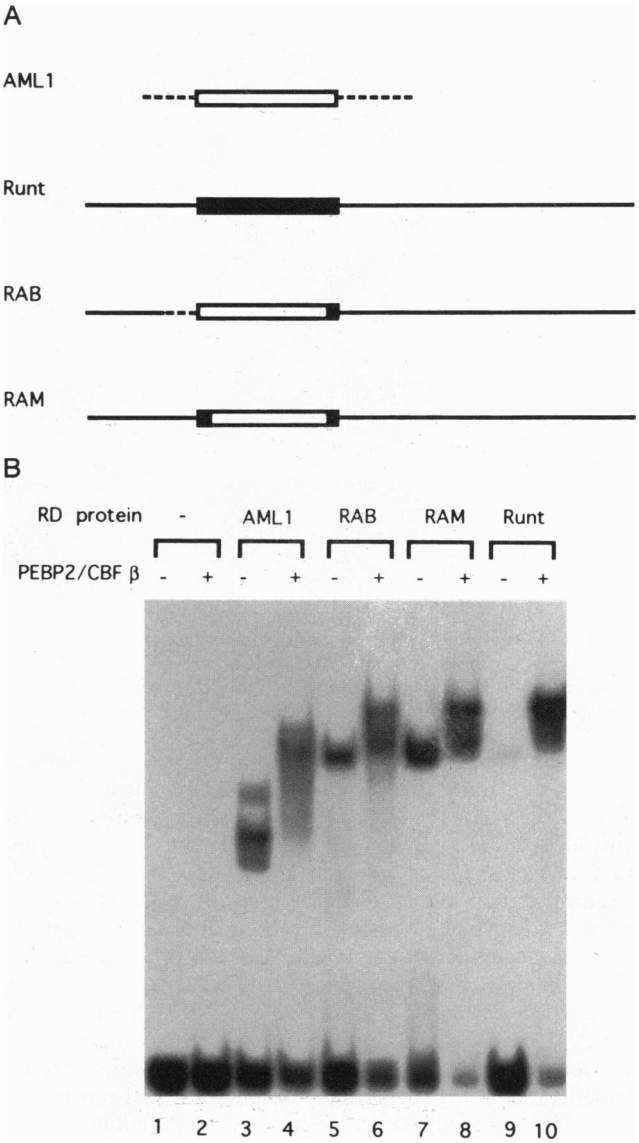

FIG. 2. DNA binding and heterodimerization activities of Runt domain proteins. (*A*) Diagrammatic representation of Runt domain and hybrid proteins. Open boxes represent Runt domain sequence from human AML1 protein. Solid boxes represent Runt domain sequence from *D. melanogaster* Runt protein. Solid line indicates sequence outside the Runt domain from Runt protein. Dashed line indicates sequence from AML1. (*B*) Electrophoretic mobility-shift assay of Runt domain-containing proteins using ^{32}P-labeled polyoma enhancer *A*-element in the absence (odd-numbered lanes) or presence (even-numbered lanes) of PEBP2/CBFβ. Lanes 1 and 2, no Runt domain protein; lanes 3 and 4, full-length AML1 protein; lanes 5 and 6, RAB hybrid protein; lanes 7 and 8, RAM hybrid protein; lanes 9 and 10, full-length Runt protein.

DISCUSSION

The overall homology of the *D. melanogaster* gene to that of *D. pseudoobscura* and *D. virilis* is consistent with interspecific studies for other genes (25–27, 33). Comparison of the Runt protein sequence from these three species identifies eight conserved blocks that account for almost all of the homology shared between these proteins. This evolutionary conservation strongly suggests that these blocks are important for *run* function. The divergence of both the sequence and the spacing between these conserved regions further suggests that these blocks define discrete functional units or modules. This information should provide a useful framework for further dissecting the functions of this regulatory protein.

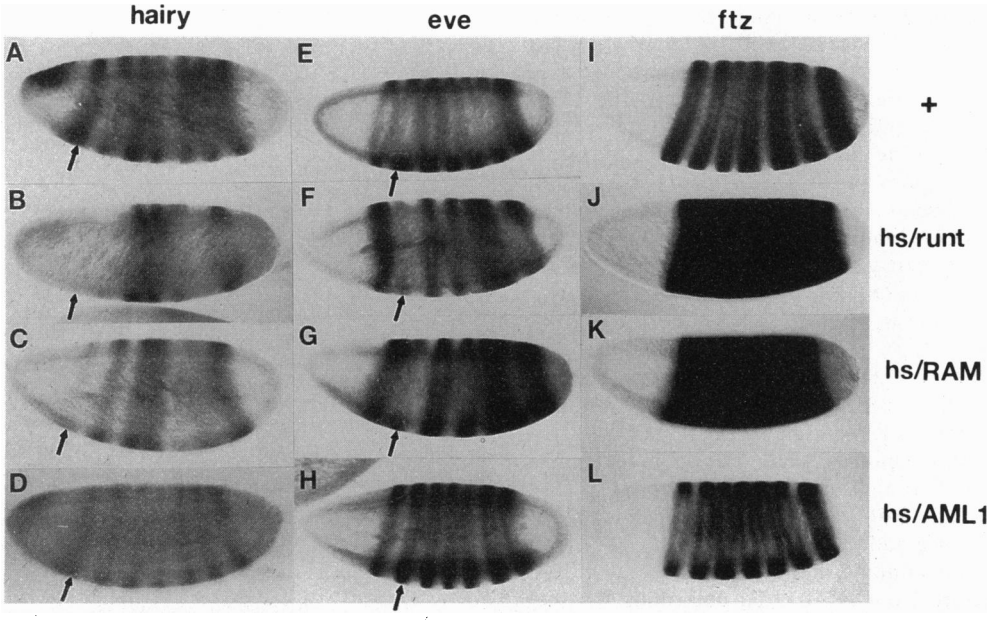

FIG. 3. Pair-rule gene expression in normal, *hs/run*, *hs/RAM*, and *hs/AML1* embryos. The pattern of *h*, *eve*, and *ftz* mRNA accumulation by *in situ* hybridization in normal (*A*, *E*, and *I*), *hs/run* (*B*, *F*, and *J*), *hs/RAM* (*C*, *G*, and *K*), and *hs/AML1* (*D*, *H*, and *L*) embryos is shown. These embryos are shown with the anterior end to the left and the dorsal side up. Arrows in *A–D* indicate position where *h* stripe 1 should form. Arrows in *E–H* indicate position where *eve* stripe 2 should form.

The limits of the Runt domain initially were determined by alignment of Runt with the human AML1 and murine PEBP2/CBFαA proteins (1). Alignment of the three *Drosophila* proteins identifies a highly conserved block that extends beyond the limits of the Runt domain at both the N-terminal and C-terminal ends. The high degree of sequence identity within this block as well as its contiguity strongly suggests that this entire block acts as a functional unit. Several other mammalian Runt domain genes have recently been isolated. This includes the murine homologue of *AML1* (11), a human homologue of PEBP2/CBFαA (referred to as *AML3*), and a human gene referred to as *AML2* (12). Alignment of these multiple protein sequences supports the original boundaries of the Runt domain. This raises questions concerning the functional significance of the conserved extensions of the Runt domain in these three *Drosophila* species. Do the homologues of a particular Runt domain gene in vertebrates (e.g., *AML1*) also share adjacent regions of high homology? The available sequence data are not informative in this regard; the AML1 proteins of mice and humans are nearly identical throughout their sequence. Analysis of *AML1* homologues in other, more divergent vertebrate species is needed to resolve this issue.

Table 1. Alteration of pair-rule gene expression patterns produced by ectopic expression of Runt domain proteins

Genotype	% embryos with alterations in gene expression		
	*h**	*eve*†	*ftz*‡
y w	**0** (90)	**0** (12)	**0** (29)
hs/run	**61** (38)	**57** (47)	**73** (15)
hs/RAM91	**11** (35)	**0** (21)	**5** (21)
hs/RAM111	**75** (24)	**52** (23)	**59** (62)
hs/AML67	**0** (17)	**0** (13)	**0** (32)
hs/AML90	**0** (8)	**0** (11)	**0** (23)

Embryos were heat shocked and then allowed to recover. Midblastoderm stage embryos were examined for alterations in pair-rule gene expression. Total number of embryos at midblastoderm stage scored is indicated in parentheses. Percentage of embryos with alterations is in boldface.
h stripe 1 missing or weak.
†*eve* stripe 2 weak or missing.
‡*ftz* stripes fused.

The Runt domain of the *Drosophila* Runt protein is 65% identical to the Runt domain of the mammalian AML1 protein. Mammalian Runt domain proteins recognize the consensus DNA binding site rACCrCa (7, 8, 34, 35). Previous experiments demonstrated that a fragment of Runt that contains the Runt domain recognized this sequence but only with low efficiency in the absence of the PEBP2/CBFβ partner protein. Here we show that the full-length protein binds to DNA in the same manner as the truncated protein. Thus, the function of conserved regions outside the Runt domain is not to augment the DNA binding activity of the Runt protein.

Experiments with chimeric proteins indicate that differences in the Runt domains of Runt and AML1 are responsible for the different DNA binding activities of these proteins. One potential explanation for this difference is that the *Drosophila* protein prefers a different binding site. However, we note that in the presence of the murine PEBP2/CBFβ protein, Runt binds as well as AML1 and the RAB and RAM hybrid proteins. Thus, the major difference between these proteins is their apparent requirement for a partner. This observation strongly suggests that the *in vivo* DNA binding activity of Runt in *Drosophila* will be extremely dependent on interaction with a partner protein homologous to PEBP2/CBFβ.

To determine whether *in vitro* differences in the Runt domain are relevant to *in vivo* function, we investigated the effects of overexpressing these proteins in *Drosophila* embryos. In this assay, the Runt–AML1 hybrid protein RAM altered expression of other pair-rule segmentation genes in a manner qualitatively similar to Runt. In contrast, AML1 appeared to be inactive. One interpretation of these results is that the function provided by the Runt domain is generic—i.e., DNA binding is important and the AML1 Runt domain is conserved enough to substitute. A second interpretation is that DNA binding by Runt is not sufficient for the transcriptional regulatory effects that are observed in *hs/run* embryos and that regions outside the Runt domain are needed to see this effect. The possibility exists that the Runt domain is not necessary for these effects. In this regard, these results may parallel those obtained in similar experiments with the *Drosophila* ho-

meodomain protein Ftz. The effects produced by overexpressing the Ftz protein do not require an intact homeodomain (36). This second interpretation is also consistent with the findings that *run*-dependent transcriptional regulation is observed with simplified reporter genes that contain binding sites for other protein factors (15, 16), presumably by mechanisms that involve protein–protein interactions. This is not to suggest that the DNA binding function of Runt is unimportant; the evolutionary conservation of the Runt domain argues otherwise. However, it is possible that the conditions used in our ectopic expression assays produce sufficient levels of protein to alter the transcriptional activity of particularly sensitive regulatory elements in the absence of a high-affinity interaction between Runt and DNA. In any case, these experiments clearly indicate that regions outside the Runt domain are important for the regulatory effects observed in *hs/run* embryos.

The conserved C-terminal pentapeptide motif VWRPY is related to the C-terminal WRPW motif that is found in a small family of related basic helix–loop–helix proteins (33, 37–40). This family includes the *Drosophila* proteins Hairy and Deadpan. Genetic experiments indicate that hairy and deadpan genes act to oppose the regulatory effects of runt on the target genes fushi tarazu and Sex lethal, respectively. In both cases, hairy and deadpan act as repressors, whereas runt is an activator. The WRPW motif is required for Hairy function and mediates interaction with the unrelated protein Groucho (33, 41). Perhaps Runt's WRPY motif mediates an interaction with Groucho that indirectly interferes with transcriptional repression by the hairy- and deadpan-encoded proteins. The WRPY motif is conserved in mammalian Runt domain proteins (10–12) but was absent in the cDNA isoform of *AML1* used in our experiments. It will be interesting to determine whether an AML1 isoform that contains a WRPY motif behaves more like Runt in our *in vivo* overexpression assay. Finally, it is provocative to note that the WRPW motif is conserved in mammalian proteins related to hairy (42–44). The evolutionary conservation of these two related C-terminal motifs suggests that the functional antagonism between Runt domain proteins and Hairy-related proteins may be an ancient aspect of metazoan development.

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